Journal of Surgical Oncology 1999;72:211–217

Adoptive Immunotherapy for Advanced Cancer Patients Using In Vitro Activated Cytotoxic T Lymphocytes

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Background and Objectives: We evaluated the clinical efficacy of adoptive immunotherapy using in vitro activated cytotoxic T lymphocytes (CTL) in the treatment of patients with advanced cancer.

Methods: CTL were induced with the mixed lymphocyte and tumor cell culture method, in which lymphocytes isolated from patient peripheral blood mononuclear cells were mixed with inactivated autologous tumor cells. Activated lymphocytes were administered intravenously to 11 patients once every 2 weeks for 10 weeks (i.e., 5 doses).

Results: Tumor reduction and decreased tumor marker were observed in 4 patients. Notably, successful CTL induction was identified in all of these patients. In patients who did not show induction of CTL response, a decreased proportion of lymphocytes, especially $CD8^+$ cells, and increased levels of $CD14^+$ cells were frequently observed. Fluorescence-activated cell sorter analysis indicated that expression of HLA class I and costimulatory factor B7-1 molecules was diminished on tumor cells. This was partly recovered with interferon-γ, which resulted in successful induction of a CTL response.

Conclusions: It was suggested that in vitro CTL induction is difficult in patients with advanced cancer. However, once the cells were induced successfully, some favorable clinical effects were seen by the adoptive transfer of such cell populations.

J. Surg. Oncol. 1999;72:211–217.

KEY WORDS: **cytotoxic T lymphocytes; adoptive immunotherapy; mixed lymphocytes and tumor cell culture; clinical trial**

INTRODUCTION

Since Mitchison [1] first reported that allo-tumor rejection was mediated by immunized lymphocytes in 1955, many clinical trials to treat human cancers by means of adoptive transfer of lymphocytes have been conducted but with little or no clinical effect [2–4]. In 1985, Rosenberg et al. [5] introduced lymphokine-activated killer cell therapy, and the reported clinical effects surprised many clinicians. However, it was later found that intravenous (iv) injection of interleukin-2 could lead to severe toxicity [6], which limited further clinical applications. In recent years, it has been widely accepted that cytotoxic T lymphocytes (CTL), originally isolated from tumor-infiltrating lymphocytes in vitro [7,8], play a major role in tumor rejection in vivo [9]. The main target antigens of CTL have been determined in malignant melanoma, and successful active immunotherapy using these antigens has been expected. There

Grant sponsor: Japanese Ministry of Education; Grant number: 10770580.

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Accepted 8 September 1999

TABLE I. Summary of Cytotoxic T Lymphocyte (CTL) Therapy for Advanced Cancer Patients

Patient no.	Age (years)	Diagnosis	Killing to autologous tumor cells[a]	CTL induction[b]	Clinical effects
1	45	Colon cancer with supraclavicular lymph node metastases	Yes	Successful	Yes
2	64	Colon cancer with multiple lung metastases	Yes	Successful	Yes
3	63	Colon cancer with peritoneal dissemination	Yes	Successful	Yes
4	45	Ovarian cancer with peritoneal dissemination	Yes	Successful	Yes
5	60	Colon cancer with multiple liver metastases	Yes	Failed	No
6	45	Colon cancer with multiple liver metastases	Yes	Failed	No
7	50	Breast cancer with multiple bone metastases	Yes	Failed	No
8	68	Colon cancer with multiple liver and lung metastases	No	Failed	No
9	62	Anal cancer with multiple liver metastases	No	Failed	No
10	45	Colon cancer with peritoneal dissemination	No	Failed	No
11	66	Bile duct cancer with multiple liver metastases	No	Failed	No

[a]Cytotoxicity ≥30% was considered to be positive cell killing (see Materials and Methods).
[b]When ≥50% of cytotoxicity was inhibited by anti-CD3 monoclonal antibody, cell killing was judged to be mediated by CTL (see Materials and Methods).

are some reports regarding CTL target antigens in adenocarcinoma [10–12], but the clinical effects using these antigens are unknown.

In the current study, we attempted to induce CTL from patient peripheral blood mononuclear cells (PBMC) using inactivated autologous tumor cells as immunogens. The clinical effects and limitations of adoptive immunotherapy using mixed-lymphocyte and tumor cell culture (MLTC)–activated lymphocytes were also examined.

MATERIALS AND METHODS

Patients

Eleven patients with advanced cancer were enrolled in this study. All patients had been nonresponsive to other conventional therapy and had clinically evaluable disease demonstrated by either physical examination or standard radiographic study. Tumors consisted of 10 adenocarcinomas and 1 squamous cell carcinoma (Table I). Nine tumors originated from digestive organs, 1 from breast, and 1 from the ovarium. All patients underwent surgery; 5 were noncurative operations, and 6 were nonresectable recurrent cases. Current therapy had been undergone for >2 months following the final therapy (e.g., surgery or chemotherapy). This study was approved by the University Ethical Committee and written informed consent obtained.

Preparation of Autologous Tumor Cells

Tumor blocks were removed aseptically from resected specimens and mechanically minced with a scalpel. To make single-cell suspensions, tumor fragments were then incubated with protease (Dispase™; Godo Susei, Tokyo, Japan) at 37°C in a 5% CO_2 incubator for 4 to 8 h. They were then passed through 200-gauge stainless-steel mesh, washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4), and resuspended in RPMI 1640 (GIBCO BRL, Rockville, MD). Some cells were resuspended in freezing medium (Cellbanker™; Dia-iatron, Tokyo, Japan) and stored in liquid nitrogen until use.

Establishment of Autologous Tumor Cell Lines

Tumor cell suspensions in RPMI 1640 supplemented with 20% fetal calf serum (Sigma, St. Louis, MO) were incubated in 25 cm^2 plastic flasks precoated with type I collagen (Iwaki Glass, Chiba, Japan) at 37°C in a 5% CO_2 incubator. Bystander cells, such as fibroblasts, were scraped out using a thin bamboo toothpick under a microscope. One colon cancer, 1 breast cancer, and 1 ovarian cancer cell line were established (Fig. 1).

Induction of CTL

CTL were induced by means of an in vitro MLTC method as previously reported [13,14]. Peripheral blood leukocytes were collected from 2,500 ml peripheral blood using an automatic leukapheresis apparatus (Cobe Laboratories, Tokyo, Japan). PBMC were diluted twice with serum-free ASF104 medium (Ajinomoto, Tokyo, Japan), overlaid onto Ficoll-Hypaque (Histopaque™, Sigma), and then centrifuged at 400 *g* for 30 min. The intermediate layer was collected, washed 3 times with D-PBS, and resuspended in ASF104 supplemented with 1% human albumin. T cell–enriched populations were collected by passage through a 3.2-gram nylon wool column (Dainippon, Tokyo, Japan), usually resulting in 2 to 4×10^8 cells. The remaining cells, adhered to the nylon wool, were mechanically separated using cool medium and added to the T cell–enriched cell population in one-fiftieth aliquots.

Approximately 2 to 4×10^6 autologous tumor cells were cultured in the presence of 100 μg/ml mitomycin C (Kyowa Hakko, Tokyo, Japan) for 1 h at 37°C. Tumor cells were then washed and used as immunogens to the

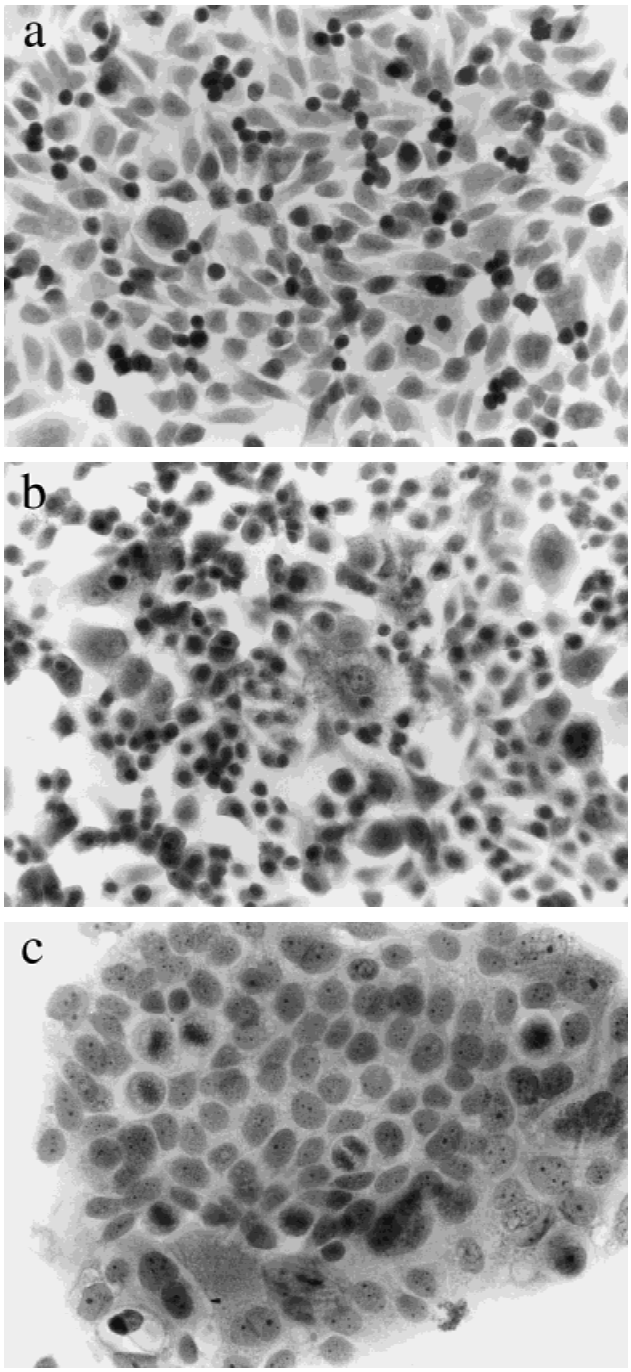

Fig. 1. Autologous tumor cell lines. Hematoxylin–eosin; original magnification 100×. **a:** CSCC, colon cancer cell line established from patient 3. **b:** CSBC, breast cancer cell line established from patient 7. **c:** CSOVC, ovarian cancer cell line established from patient 4.

reconstituted lymphocytes. MLTC was performed for 7 days in ASF104 medium supplemented with 1% human albumin. On day 1, recombinant interleukin-4 (Dainippon) at 10 U/ml was added, to inhibit lymphokine-activated killer cell induction [15], and recombinant interleukin-2 (Shionogi, Osaka, Japan) at 400 IU/ml was added on day 3. At the end of the culture period, live cells were collected by Ficoll-Hypaque density-gradient centrifugation, washed, and used as effector cells.

Cytotoxicity Assay

The cytotoxicity of activated lymphocytes against autologous tumor cells was measured using a Cytotox 96™ (Promega, Madison, WI) nonradioactive assay kit. Assays were performed in quadruplicate using 96-well round-bottomed plates. Four different sets of cell mixture were prepared: in experimental wells (E), activated lymphocytes at 4×10^5 cells/well were mixed with 2×10^4 target cells/well in a total volume of 200 μl ASF104 medium supplemented with 1% human albumin; effector spontaneous (ES) wells contained only lymphocytes at 4×10^5 cells/well in 200 μl of medium; target spontaneous (TS) wells contained only target cells at 2×10^4 cells/well in 200 μl of medium; maximum target (MT) wells contained target cells at 2×10^4 cells/well in 180 μl of medium. Plates were first centrifuged at 250 *g* for 4 min, then incubated at 37°C in a 5% CO_2 incubator for 12 h. At 45 min prior to termination of the culture, 20 μl of lysis solution (9% w/v Triton™ X-100) was added to the MT wells. Plates were then centrifuged at 250 *g* for 4 min, and 50 μl aliquots of the supernatants were transferred to 96-well flat-bottomed assay plates. The substrate mixture to the lacticate dehydrogenase released from dead cells was added to each well. Absorbance (OD, optical density) from the final red formazan products was measured at 490 nm using an enzyme-linked immunosorbent assay reader. Cytotoxicity was measured according to the following formula: % cytotoxicity = OD (E – TS – ES)/OD(MT – TS) × 100. More than 30% cytotoxicity against autologous tumor cells was judged as positive cell killing.

CTL Assay

At the beginning of the cytotoxicity assay, mouse anti-CD3 IgG monoclonal antibody (MAb) was added at a final concentration of 10 μg/ml in experimental wells, and wells were examined for alteration of cytotoxicity. When observed cytotoxicity was inhibited by the anti-CD3 MAb to more than half of the control value, cell killing was considered to be CTL-mediated.

Tumor Cell Lines

Gastric cancer cell lines GC121288 (HLA A2/A26) and GC022588 (HLA A2/A24) were kindly provided by Dr. S. Fujimoto (Kochi Medical College, Nangoku, Japan). WiDr (HLA A24), LoVo (HLA A11), and SW620 (HLA A2) cells were purchased from the ATCC (Rockville, MD).

Flow Cytometry

Expression of HLA class I and CD80 molecules on tumor cells was investigated by flow cytometry. MAbs

used were anti-HLA class I (W6/32; Dako, Copenhagen, Denmark) and anti-CD80 (BB1; Ancell, Bayport, MN).

Administration of CTL to Patients

After MLTC, lymphocytes were suspended in 20 ml saline and administered iv to patients regardless of the in vitro CTL activity. Just before lymphocyte transfer to the patients, stains (Gram's or Wright's) were examined to detect bacterial or tumor cell contamination. If there was the slightest possibility of contamination, the lymphocytes were discarded. A 2-week cycle of MLTC lymphocyte administration was repeated, and clinical effects were evaluated with tumor markers and standard radiographic studies after 5 cycles. The adverse reaction inherent to this therapy was carefully assessed by the standard evaluation of toxicity proposed by the World Health Organization.

RESULTS

Induction of CTL and Clinical Effects

The results of in vitro CTL induction and the clinical effects in patients are summarized in Table I. Overall, slight fever in 3 patients was noted as a measurable side effect; no other significant adverse reactions were noted during the therapeutic period or for 3 months afterward. Among the 11 patients evaluated, 4 (patients 1–4) exhibited in vitro CTL activity at least once during the series of 5 MLTC lymphocyte administrations. Although in vitro tumor cell killing was also seen in some of the remaining 7 patients, cytotoxic activity was not inhibited by anti-CD3 MAb, suggesting that the killing was $CD3^+$ cell (i.e., CTL)–independent (Table I). No clinical efficacy was observed in these 7 patients. In contrast, in the 4 patients who showed in vitro CTL activity at least during the trial period several clinical effects were observed. In patient 1, the metastatic supraclavicular lymph nodes shrank from 5.5 cm in diameter to 3.5 cm, which remained consistent for 2 months (Fig. 2a). Serum carcinoembryonic antigen (CEA) levels also decreased to half of the pretreatment levels. In patient 2, a colon cancer patient with rapidly growing multiple lung metastases, metastatic growth stopped for >2 months (Fig. 2b). In patient 3, a colon cancer patient with peritoneal dissemination, rapidly increasing serum CEA levels remained at a plateau for 2 months after treatment (Fig. 2c). In patient 4, an ovarian cancer patient, massive ascites caused by tumor dissemination diminished after iv and intra-abdominal injections of activated lymphocytes; serum CA125 levels also decreased (Fig. 2d).

Lymphocyte Subset and CTL Activity

PBMC subsets in the pretreatment period are shown in Figure 3. There was no difference in natural killer cell activity or in the percentage of peripheral $CD3^+$ T cells between patients who showed CTL activity in vitro and those who did not. In contrast, decreased $CD8^+$ cell ratios together with an increase of $CD14^+$ cell levels were commonly observed in patients who did not show CTL activity (Fig. 3).

Expression of HLA Class I and B7-1 on Tumor Cells

Expression of HLA class I and B7-1 (CD80) molecules on tumor cells was analyzed by a fluorescence-activated cell sorter (Table II). Among the 3 cancer cell lines established from patient tumors, 1 breast tumor cell line, originated from patient 7, showed diminished expression of HLA class I. None of them expressed B7-1. In addition, among 5 other cancer cell lines examined, 1 colon cancer cell line, LoVo, showed decreased HLA class I expression. All of these cell lines were negative for B7-1 expression.

When these tumor cells were cocultured with 1 ng/ml interferon-γ (IFN-γ) for 1 week [16,17], expression of HLA class I was recovered in 1 autologous and 1 irrelevant cancer cell line that originally showed diminished expression (Table II). B7-1 expression was partly recovered in 2 autologous and 1 irrelevant cancer cell lines (CSOVC, CSCC, and SW620; Table II). Thus, using activated autologous cell lines as immunogens, in vitro CTL activity was successfully induced in patient 4, in whom no other CTL activity was seen.

DISCUSSION

In the current report, we evaluated the clinical efficacy of adoptive immunotherapy using lymphocytes activated with the MLTC method. We also examined factors preventing the successful induction of CTL. To date, impaired antitumor immunity in patients with advanced cancer, especially related to the production of tumor-specific CTL, has been attributed mainly to a deficit of $CD4^+$ cell function, while antigen-presenting cells are able to express tumor antigens on HLA molecules [18]. This deficit in $CD4^+$ cell function is reported to be reversible [19] and may be mediated by some soluble factors produced by tumors, such as transforming growth factor-β [20]. To eliminate such negative factors for the induction of tumor-specific CTL, we utilized an in vitro MLTC method in which inactivated autologous tumor cells were used as immunogens at an optimal ratio to patient PBMC [13,14]. In 4 of 11 patients, CTL were induced using this protocol, and various degrees of clinical response were observed. In contrast, there were no clinical responses in the remaining 7 patients, who did not exhibit in vitro CTL activity. These findings suggested that, upon successful induction of specific CTL, clinical response of adoptive immunotherapy could be expected even in patients with advanced carcinomas.

In recent years, various specific tumor-associated antigens, expressed on HLA class I molecules, have been

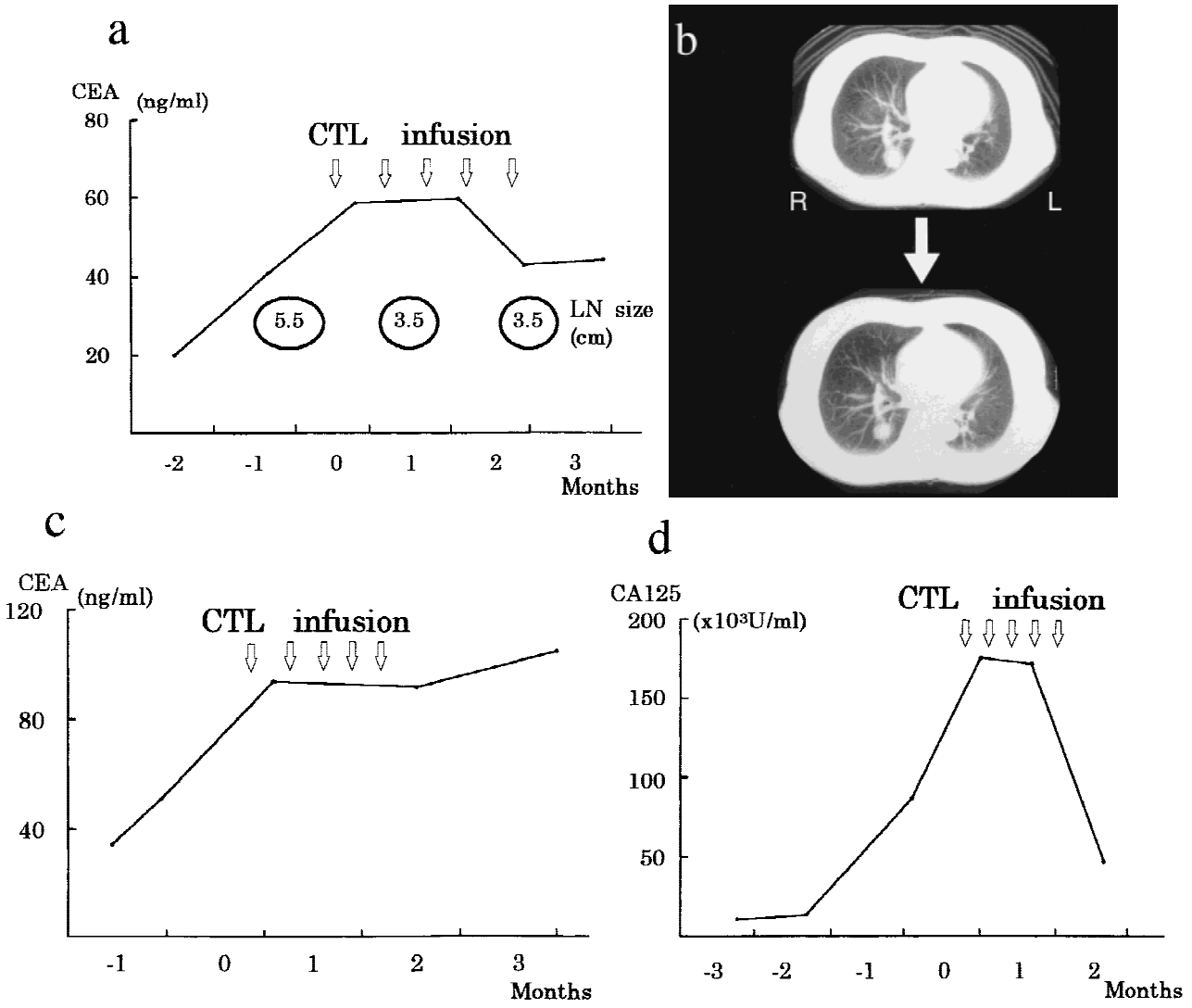

Fig. 2. Clinical effects of the cytotoxic T lymphocyte (CTL) therapy. **a:** Patient 1. Serum carcinoembryonic antigen (CEA) levels and the size of the metastatic supraclavicular lymph node (LN) are shown. **b:** Patient 2. The rapid growth rate of a lung metastasis was inhibited for 2 months (arrow). **c:** Patient 3. Rapidly increasing serum CEA levels were reduced. **d:** Patient 4. Massive ascites was diminished by intra-abdominal and intravenous injection of activated lymphocytes. Rapidly increasing serum CA125 levels were also decreased (arrows).

TABLE 2. Tumor Cells Expressing HLA class I and B7-1

Tumor cell lines	Origin	Class I HLA (%)		B7-1 (%)	
		Naive	IFN-γ-treated	Naive	IFN-γ-treated
Autologous					
CSBC	Breast cancer (patient 7)	13	97	<5	<5
CSOVC	Ovarian cancer (patient 4)	≅100	≅100	<5	18
CSCC	Colon cancer (patient 3)	≅100	≅100	<5	15
Irrelevant					
GC121288	Gastric cancer	≅100	≅100	<5	<5
GC022588	Gastric cancer	≅100	≅100	<5	<5
WiDr	Colon cancer	≅100	≅100	<5	<5
LoVo	Colon cance	63	90	<5	<5
SW620	Colon cancer	≅100	≅100	<5	50

HLA class I expression was reduced in naive CSBC and LoVo cell lines and recovered by treating them with interferon (IFN)-γ. B7-1 was not expressed on any of the naive cell lines tested. A slight increase of B7-1 was observed in CSOVC, CSCC, and SW620 cell lines after IFN-γ culture (1 ng/ml of IFN-γ for 7 days). The positive ratio was calculated with Kolgomolov-Smirnov statistics.

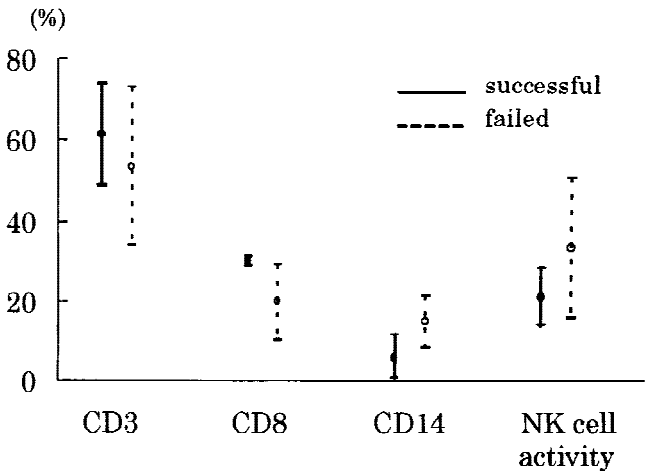

Fig. 3. Pretreatment immunological parameters and cytotoxic T lymphocyte induction. NK, natural killer.

identified [10–12]. These antigens are expected to be utilized in both adoptive and active immunotherapies against cancer. However, the clinical efficacy of immunotherapies directed against single antigens in treating carcinomas, which usually consist of heterogeneous components, remains to be clarified. The MLTC method may not be an effective or reproducible approach for CTL induction; however, when the cells are appropriately prepared, there is a possibility of generating CTL to several different tumor-associated antigens at the same time. For this reason, CTL induction using autologous tumor cells as immunogens should be stressed more, and a further effort to improve the successful induction ratio is necessary.

In the current study, there was a tendency for CTL not to be induced from patients with low $CD8^+$ or high $CD14^+$ cell populations in the peripheral blood, which is consistent with other reports [13,21]. While the precise mechanisms responsible for this are unknown, the results suggested that host factors play an important role in the inhibition of in vitro CTL induction.

Meanwhile, tumor cell factors have also been suggested to contribute to the inhibition of CTL induction. Some of the autologous cells and established tumor cell lines showed diminished HLA class I expression on the cell surface. When these cell lines were used as immunogens, CTL were never induced. In addition, there was little to no expression of the costimulatory factor B7-1 on any of the cells tested.

When these cells were stimulated with IFN-γ, some cell lines expressed B7-1 on the surface to various degrees. In 1 patient from whom a cancer cell line was established, CTL was successfully induced using IFN-γ-treated autologous tumor cells, whereas otherwise no CTL activity was observed (patient 4). These findings also suggested the major contribution of tumor side factors to in vitro CTL induction. How these negative factors, originating from both host and tumor, can be overcome remains to be resolved.

Adoptive immunotherapy using in vitro activated CTL had some favorable clinical effects with little or no side effects. This study provided basic information as to the success ratio of in vitro CTL induction and the clinical effects of patients treated with activated CTL.

In conclusion, the current data suggest that in vitro CTL induction is difficult and affected by various negative factors of both host and tumor origin. However, once the cells are induced successfully, some favorable clinical effects may be anticipated, even in patients with advanced cancers.

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